Sequence Analysis of a cDNA for Lysyl Hydroxylase Isolated from Human Skin Fibroblasts from a Normal Donor: Differences from Human Placental Lysyl Hydroxylase cDNA

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Using polymerase chain reaction, we have isolated and sequenced a 3-kb cDNA for lysyl hydroxylase (LH) from human skin fibroblasts from an normal donor. Apart from two polymorphic sites, no differences were observed between the 2184 nt coding regions of LH cDNA from fibroblasts and placenta. However, four differences were observed in the 3′ non-coding regions of the two cDNAs; three were single base changes and the fourth a deletion of a single base. The absence of the single nucleotide in the LH cDNA from fibroblasts resulted in the loss of an HpaII site that is present in the placental LH cDNA; this was confirmed in HpaII digests of fibroblast and placental LH cDNAs from the same donor. Northern blots showed that the LH gene was strongly expressed in fibroblasts and placenta and, to a lesser extent, in aorta, lung, vein, cartilage, and artery. *Key words: collagen biosynthesis / hydroxylysine / polymorphism / Ehlers-Danlos syndrome type VI. J Invest Dermatol 102:382–384, 1994*

Lysyl hydroxylase (LH) is an important enzyme in collagen biosynthesis that catalyzes the formation of hydroxylysine, which is essential for the intermolecular crosslinking of collagen [1]. The requirement for LH is demonstrated in patients with Ehlers-Danlos syndrome type VI (EDS VI), a connective tissue disorder characterized by decreased levels of LH activity [2,3]. The severity of symptoms in these patients varies greatly and does not necessarily correlate with the same degree of impaired hydroxylation [4]. Although the biochemical properties of LH from various tissues synthesizing different collagen types are remarkably similar [5], the variability in the types of collagens that are hydroxylated and the localization of underhydroxylated collagens in specific tissues suggests that a family of LH isozymes may exist, each with its own specificity in different tissues for different collagen types [6,7].

We have recently reported the sequence of a partial cDNA (2.2 Kb) for LH from dermal fibroblasts from a normal donor [8], which has been completed in the present study. A comparison of our fibroblast LH cDNA sequence with the recently published sequence of human placental LH cDNA [9] shows several single base changes, two of which appear to be polymorphisms, and one single base deletion that results in the loss of an HpaII site in the 3′ non-coding region. In addition, we have examined expression of the LH gene in different tissues by Northern blot hybridization of total RNA to our 2.2 Kb cDNA for LH.

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Abbreviations: LH, lysyl hydroxylase; Kb, kilobase pair; nt, nucleotide; EDS VI, Ehlers-Danlos Syndrome type VI.

MATERIALS AND METHODS

Cell Strains and Tissues The fibroblast cell strains were from a normal 14-month-old boy (GM05659), a normal newborn boy (AG01519A) (both strains obtained from the Institute for Medical Research, Camden, NJ), and an explant from a skin biopsy from a normal female donor (AMS1106). Placental tissue was obtained from the same donor (AMS). Tissue samples of aorta, diaphragm, dura, esophagus, gall bladder, lung, vein, spleen, cartilage, artery, and intestine were obtained from another donor (EH).

RNA Isolation

Dermal Fibroblasts: Total RNA was isolated from approximately 6×10^6 fibroblast cells, by the acid-guanidinium thiocyanate-phenol-chloroform extraction method as previously described [8]. Poly (A) RNA was prepared using oligo (dT)-cellulose columns [8].

Human Tissue: Each tissue sample was pulverized with a pestle and mortar, and cooled with liquid nitrogen; total RNAs were isolated as described for fibroblast cells using 5.4 ml of guanidinium thiocyanate per 1 g tissue.

Northern Blot Analysis: Total RNAs (9 μg) from fibroblasts, placenta, and other human tissues with the exception of cartilage, artery, and intestine (6 μg RNA) were separated electrophoretically and blotted [8]. The blots were hybridized with the random-primed ^{32}P-labeled 2.2-Kb cDNA probe for LH as previously described [8].

Isolation, Amplification, and Sequencing of a cDNA for LH We have recently described the amplification and sequencing of a partial cDNA for LH from dermal fibroblasts (GM05659) encompassing a 2.2-Kb sequence from nt 760 to nt 2939 preceding the poly (A) tail. The approximately 800 bases remaining at the 5′ end of the LH cDNA were amplified by a 5′ RACE technique [10] (Gibco-BRL). First-strand cDNA was synthesized from poly $(A)^+$ RNA isolated from dermal fibroblasts using, as primer, the sequence complementary to nt 898–917 of LH cDNA and reverse transcriptase. The 5′ end of the cDNA was dC-tailed using dCTP and terminal deoxynucleotidyl transferase. This was followed by PCR amplification using, as 3′ amplification primer, the nested sequence complementary to nt

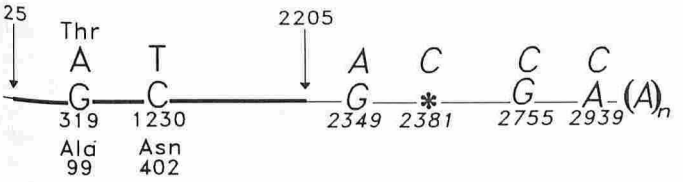

Figure 1. Sequence differences between LH cDNAs from human skin fibroblasts and placenta. The *line* represents the sequence of LH cDNA from human skin fibroblasts (not shown to scale). The *vertical arrows* at nts 25 and 2205 delineate the start and stop of the coding region *(thicker line)*; the nts in the placental LH cDNA that differ from fibroblast cDNA are shown above the *line*. The only predicted aa change is a Thr (placenta) for Ala_{99} (fibroblast). The *asterisk* represents the loss of C_{2381} in fibroblast LH cDNA. The numbering is based on the sequence of fibroblast LH cDNA, GenBank accession number, M98252.

808–827 and as 5′ amplification primer, an anchor primer containing a poly (dG) region. The amplified sequence was gel-purified and cycle-sequenced (Gibco-BRL) in both directions. The sequence differences in this cDNA from the LH cDNA sequence of human placenta [9] were confirmed by sequence analysis of these regions in a fibroblast LH cDNA isolated from another donor (AG01519A).

Restriction Digests with HpaII First-strand cDNA was synthesized from poly $(A)^+$ RNA isolated from fibroblasts and placenta from the same donor (AMS). Amplification of the 1326-bp region covering the HpaII difference at nt 2381 was carried out using as 5′ primer the sequence nt 1497–1515, and as 3′ primer the sequence complementary to nt 2805–2823. Polymerase chain reaction amplification was performed under previously described conditions [8]. The gel-purified (Glassmax; Gibco-BRL) 1326-bp fragments from fibroblasts and placenta were digested with HpaII. The digests were electrophoresed on a 3% NuSieve–1% agarose gel and visualized by ethidium bromide–UV fluorescence.

RESULTS AND DISCUSSION

The 2939-bp cDNA for LH from human skin fibroblasts has an open reading frame of 2184 nts coding for a 727–amino acid polypeptide of similar size to that previously reported for the LH cDNA from human placenta [9]. There are some differences in the fibroblast LH cDNA sequence from the placental sequence as shown in Fig 1; in the coding region, however, the changes of G_{319} for A ($Ala_{99} \rightarrow$ Thr) and C_{1230} for T (conserved Asn_{402}) appear to be at polymorphic sites. The change of G_{319} for A has been reported by Hautala *et al* [11] in some of their placental clones, and also we have observed in our recent sequence analysis of two mutant LH alleles in fibroblasts from an EDS VI patient (Ha *et al,* manuscript submitted) that one allele had an A at nt 319, again indicative of polymorphism. In addition, the presence of a T at nt 1230 in the other allele suggests that this site is also polymorphic.

In the 3′ non-coding region the fibroblast sequence differs from the placental sequence by three single base changes as shown in Fig 1. In addition, the fibroblast sequence lacks a C between nt 2380 and nt 2381 that is present in the placental sequence and results in the loss of an HpaII site. We confirmed the loss of this HpaII site by electrophoresis of HpaII restriction digests of a 1326-bp polymerase chain reaction–amplified region of fibroblast and placental cDNA isolated from the same donor (Fig 2*A*). This showed that the 912-bp fragment from the fibroblast digest replaced the two fragments (470 bp and 442 bp) from the placental digest (Fig 2*B*), thus verifying the loss of this HpaII site in fibroblast cDNA. As the cDNAs were obtained from skin fibroblasts and placenta from the same donor, this would indicate that this is not a simple polymorphic variant but a true difference between the LH cDNAs isolated from these tissues.

We have previously observed [8] that the 2.2-Kb LH cDNA probe hybridizes with two mRNAs of 2.4 and 3.4 Kb on Northern blots of total RNA isolated from fibroblasts. This probe hybridized strongly to Northern blots of total RNA (Fig 3) isolated from placenta *(lane 7)* and skin fibroblasts *(lane 10)*. The LH gene was also

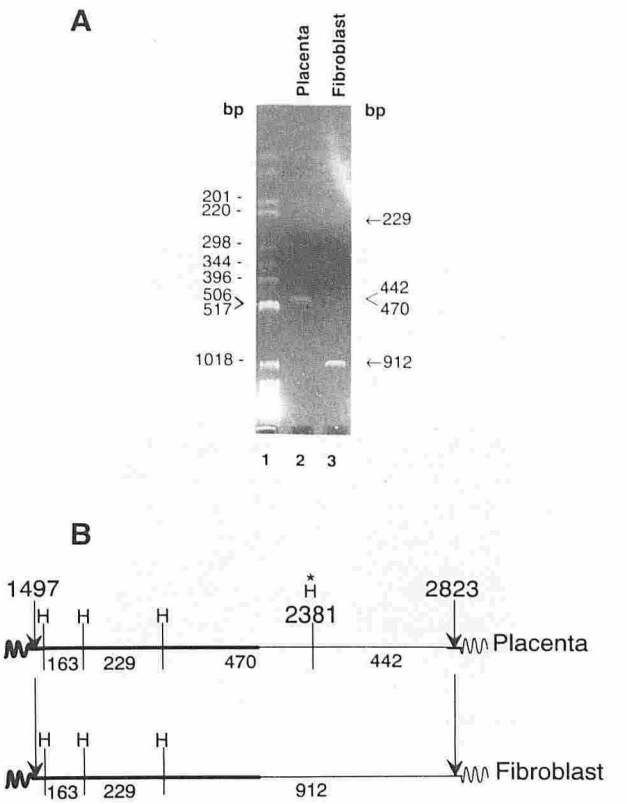

Figure 2. Electrophoresis of HpaII digests of polymerase chain reaction–amplified sequences from fibroblast and placental cDNAs showing loss of an HpaII site in fibroblast cDNA. *A)* A 1326-bp region in the LH cDNAs from fibroblast and placenta was amplified by polymerase chain reaction and digested with HpaII. Ethidium bromide staining of the digests electrophoresed on a 3% NuSieve–1% agarose gel (major fragments identified by *arrows*) showed that the largest-sized fragment from the fibroblast digest *(lane 3)* was approximately twice the size of the largest fragments from the placental digest *(lane 2)*. *Lane 1* represents electrophoresis of 2 μg of a 1-Kb DNA ladder with the sizes marked as shown. *B)* Location of the HpaII restriction sites (H) in the amplified sequences of fibroblast and placental LH cDNAs between nt 1497 and 2823 *(vertical arrows)*. The site at nt 2381 present in placental cDNA but absent in fibroblast cDNA is marked with an *asterisk*. Fragment sizes in base pairs are marked below the lines. The larger 912-bp fragment from the fibroblast digest observed in the gel electrophoresis *(A)* confirms the loss of the HpaII site at nt 2381. The coding and noncoding sequences are represented by *thicker* and *thinner lines,* respectively.

expressed, although to a lesser extent, in other human tissues including aorta, lung, vein, cartilage, artery, and to a lesser extent spleen and gall bladder. Although the LH mRNAs were variably expressed in these tissues, they appeared to be of identical sizes. Little or no LH mRNA was detected in diaphragm, dura, esophagus, or intestine. Other studies have shown considerable expression of the mRNAs for LH in endothelial cells and, to a lesser extent, in keratinocytes (data not shown).

In summary, sequencing of a cDNA for LH isolated from dermal fibroblasts has shown some differences from the placental cDNA. Although certain of these changes may represent simple polymorphic variants, a true difference in the 3′ noncoding region between fibroblast and placental LH cDNA has been identified by HpaII restriction digests. Whether these differences are the result of alternative splicing of RNA from the same gene, are the products of different genes, or occur via another mechanism remains to be determined; however, such studies should yield some insight into variables such as the phenotypic differences of LH-deficient EDS VI individuals and the tissue specificity of lysyl hydroxylation.

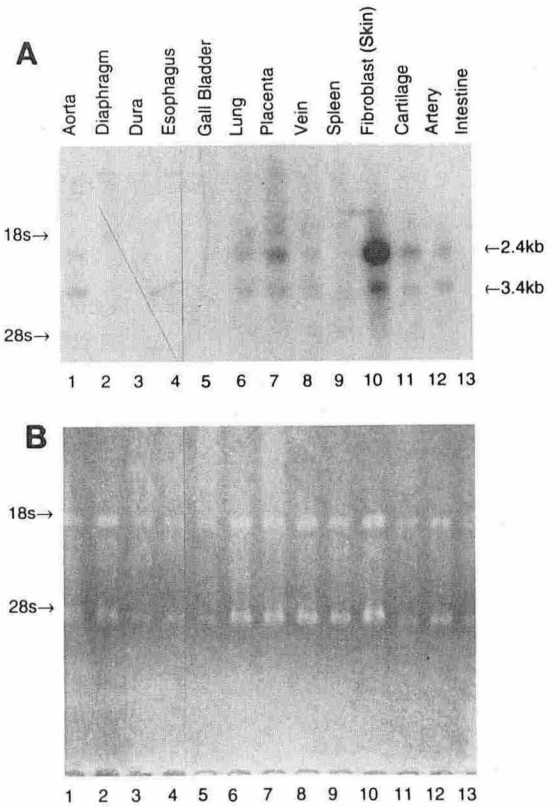

Figure 3. Northern blot showing expression of LH in human tissues. *A)* Total RNA was isolated from fibroblasts and several human tissues as described in *Materials and Methods.* Nine micrograms total RNA *(lanes 1–10)* and 6 μg total RNA *(lanes 11–13)* were electrophoresed and transferred to GeneScreen Plus membrane, and the blot was hybridized with the 2.2 Kb cDNA probe for LH (which detects both 2.4 and 3.4 Kb mRNAs), as described. *B)* The uniformity of loading of RNA samples was assessed by comparison of the rRNAs (18S and 28S) detected by ethidium bromide staining and UV fluorescence.

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